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Oxidative stress of sea slugs under tidal emersion

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Dissertação apresentada à Universidade de Lisboa para cumprimento dos requisitos necessários â obtenção do grau de Mestre em Ciências do Mar, realizada sobre a orientação do Doutor Rui Afonso Bairrão da Rosa, Investigador Principal do MARE – Laboratório Marítimo da Guia, da Faculdade de Ciências da Universidade de Lisboa.

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Resumo

Os habitats intertidais são ambientes bastante instáveis devido às flutuações cíclicas de marés. Estas zonas são afectadas principalmente pela temperatura, salinidade, variações da radiação ultravioleta, exposição ao ar e acção das ondas. Durante os ciclos de marés, os organismos sofrem dissecação e stress oxidativo, tendo como consequência uma alteração na estrutura das proteínas e perda da função celular. Em condições de stress os organismos produzem espécies reactivas de oxigénio como mecanismo de defesa. Contudo, se esta produção for excessiva pode danificar ou levar à morte celular. Os biomarcadores indicam as respostas a stress físicos ou químicos, quantificam os produtos secundários produzidos ou inibidos pelos organismos durante o período de exposição ao stress.

O stress oxidativo, desequilíbrio entre a produção de espécies reactivas de oxigénio e a respectiva eliminação através de sistemas biológicos que as removam ou reparem os danos causados por elas, leva os organismos a produzirem espécies reactivas de oxigénio, e consequentemente à incapacidade dos organismos para se desintoxicarem das mesmas. As espécies reactivas de oxigénio desempenham um papel fundamental na manutenção da actividade celular, porém a produção excessiva pode provocar danos. Por exemplo, as espécies reactivas de oxigénio ao reagirem com os lípidos das membranas provocam peroxidação lipídica. Certos organismos aquáticos possuíam uma estratégia de defesa, "preparação para o estado de stress oxidativo", com o intuito de minimizar os efeitos colaterais do stress oxidativo. Contudo, este fenómeno ainda não foi observado em lesmas do mar, nomeadamente em nudibrânquios. Ainda é desconhecido se a reoxigenação causa danos a nível celular nestes organismos. Os nudibrânquios são organismos bastante atractivos pelo seu manto bastante colorido e diversificado, e pela particularidade de possuírem as brânquias na parte exterior do corpo e em volta do ânus. As lesmas do mar realizam migrações de zonas subtidais para zonas intertidais como parte essencial para o ciclo reprodutor, ficando exposto a factores ambientais bastante stressantes para a sua sobrevivência, como a disponibilidade de oxigénio. Estes organismos têm modificações anatómicas e comportamentais alternativas que permite difundir o oxigénio através do corpo, complementam o consumo de oxigénio através de respiração cutânea, possuem um manto bastante irrigado que lhes permite realizam trocas gasosas com o meio ambiente.

O presente estudo teve como objectivo analisar as expressões das proteínas de choque térmico, peroxidação lipídica (indicador de dano celular) e a actividade das

enzimas antioxidantes - superóxido dismutase, catalase e glutationa S-transferase) de duas espécies de nudibrânquios, *Dendrodoris grandiflora* e *D. herytra*, de modo a compreender se as duas espécies possuem estratégias defensivas preparatórias para o stress oxidativo durante períodos de emersão. No local de amostragem, observou-se que estes organismos preferem substrato irregular, com fendas, sombras, ou fissuras com água retida que lhes oferecem abrigo para os períodos de exposição ao ar. Em laboratório, verificou-se que as proteínas de choque térmico aumentaram significativamente, obtendo um valor máximo de 10.32 ng^{-1} mg por proteína total aos 60 minutos de emersão. A glutationa S-transferase apresentou uma tendência para aumentar a sua expressão durante a fase de emersão, obtendo valores de 36.46 $nmol^{-1}$ min^{-1} mg^{-1} por proteína total. Contudo, em todas as expressões enzimáticas verificou-se que durante o período de reoxigenação a tendência seria diminuir. Assim, quando um organismo é exposto a ambiente com baixos níveis de oxigénio, os organismos começam a produzir espécies de oxigénio reactivas causando peroxidação lipídica podendo activar o factor de transcrição Nrf2 de modo a desencadear o mecanismo de preparação para o stress oxidativo. E ainda, possuem a capacidade de diminuir o seu metabolismo quando se encontram em ambientes com condições pobres em oxigénio, ou seja, conseguem reduzir o consumo de energia e actividade enzimática durante a privação de oxigénio. Esta estratégia defensiva é reforçada por comportamentos mecânicos, os organismos tentam refugiarem-se em locais menos expostos directamente à radiação solar, de modo a minimizar os impactos biológicos.

Palavras-chave

Enzimas antioxidantes; Proteínas de choque térmico; Respostas de defesa; *Dendrodoris grandiflora*; *D.herytra*;

Abstract

The intertidal habitats are mainly affected by temperature, salinity, changes in ultraviolet radiation, air exposure and wave action. During tidal cycles, these organisms undergo desiccation and oxidative stress, causing changes in protein structure and loss of cellular function. Biomarkers indicate physical or chemical stress responses, quantify the side products produced or inhibited by organisms during the stress exposure period.

Oxidative stress, the imbalance between the production of oxygen reactive species and their elimination by biological systems that remove or repair the damage caused by them, lead the organisms to produce oxygen reactive species, and thus the inability of the body to detoxify them. Oxygen reactive species play an important role in maintaining the cell activity, but excessive production may cause damage, for example, lipid peroxidation. This Lipid peroxidation may interrupt the functionality of the membrane and deactivate proteins. Some aquatic organisms had a defense strategy, "preparation for the state of oxidative stress", in order to minimize the side effects of oxidative stress. However, this phenomenon has not been observed in sea slugs, in particular nudibranchs. The nudibranchs are very attractive organisms for its very colorful and diverse mantle, and by having the gills outside their body and around the anus. Target species of this study are *D.grandiflora* and *D. herytra*, subtidal species that live in rocky and uneven substrates commonly associated with corals and sponges.

This study aimed to quantify of heat shock proteins, lipid peroxidation (indicator of cellular damage) and activity of antioxidant enzymes (superoxide dismutase, catalase and glutathione S-transferase) to understand if these two nudibranch species have preparatory defensive strategies to oxidative stress. The samples were retrieved in an oyster bank located in Mitrena, Sado Estuary Natural Reserve. Our data revealed that when an organism is exposed to an environment with low oxygen levels, the organism starts to produce reactive oxygen species causing lipid peroxidation, which can activate the Nrf2 transcription factor to trigger the preparation mechanism to oxidative stress. And also have the ability to decrease their metabolism when they are in environments with poor conditions of oxygen, ie, they can reduce the consumption of energy and enzymatic activity during oxygen deprivation. This defensive strategy is reinforced by mechanical behavior, organisms trying to take refuge in locations less exposed directly to solar radiation so as to minimize the biological impacts.

Keywords

Antioxidant enzymes; Heat shock proteins; Defense responses; *Dendrodoris grandiflora*; *D. herytra*;

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1-Introduction

1.1- Intertidal regions

The coastal aquatic habitats and estuary areas are extremely productive sites, having a large biodiversity (Matoo *et al.,* 2013). But they are also unstable concerning abiotic parameters (Almeida and Bainy, 2006). Intertidal environments (marine and dry land habitat borders) are characterized by cyclical fluctuations induced by fluctuations of the sea level, sun and moon gravitational attraction, and centrifugal forces due to earth rotation (Freire *et al.,* 2011; Gracey *et al.,* 2008; Nybakken, 1988; Willmer *et al.,* 2005).

The main environmental stressors are temperature, dissolved oxygen concentration, salinity, ultraviolet (UV) radiation fluctuations, pH, dissection by air exposure, wave dynamics and hypoxia (Teixeira *et al.,* 2013). Temperature and dissection vary on a season and daily basis, according to the tidal cycle and climate condition of the environment (Dong *et al.,* 2008). These two are the most important parameters, because of their essential role in the invertebrate development (Sisson, 2005).

During low tide, the organisms living in upper intertidal areas are emersed, laying exposed to waves, dissection and strong rain and UV (Freire *et al.,* 2011). These organisms may suffer changes in biochemical, physiological and molecular processes during homeostasis (Madeira *et al.,* 2013).

1.2- Biomarkers

According to Gestel and Brummelen (1996), biomarkers are individual environmental stress biological response indicators, quantifying secondary products of an organism when stimulated that way. In other words, stress may inhibit or stimulate endogenous enzymatic activity, but the way enzymes express themselves will depend on time of exposure, concentration of a compound that is being monitored, and the particular life state of the organism being studied (initial stages of development are more sensitive to stress).

Stress responses, such as regulation of temperature dependent gene expression, allow marine organisms to adapt or successfully acclimate to new environments (Yamashita *et al.,* 2010). Some organisms have cells that show transient resistance to high temperature induced heat (Ciavarra *et al.,* 1994). This phenomenon known as

acquired thermos tolerance is associated to a quick synthesis of an amount of highly preserved protein, heat shock proteins (HSPs) (Pelham, 1986).

The high fluctuation in the above mentioned environmental parameters, such as oxidative stress leads the organisms to produce Reactive Oxygen Species (ROS) and to the organism inability to detoxify active ROS or repair injury (Halliwell, 1994). This compound results from the incomplete reduction of oxygen, and the production of peroxide species (Regoli, *et al.,* 2002). ROS production in aquatic environments is quite common (Lesser, 2011), they are naturally synthesized and have an important role in the maintenance of inter and intercellular cell activity. When the organisms are exposed to intensive environmental stress, ROS levels may increase drastically and cause damage to cells (for example, cytoskeleton, mitochondria, proteins, lipids and DNA) (Halliwell and Gutteridge, 1999; Regoli *et al.,* 2002), so the formation of ROS during heat stress results from hyperthermia (Freire *et al.,* 2011), and tissue reoxygenation during recovery (Halliwell and Gutteridge, 1999). But at the same time, ROS may react with lipids of the organisms, specifically membrane lipids, causing lipid peroxidation, one of the most usual cell damage (Lesser, 2011). Lipid peroxidation may interrupt membrane functionality, deactivate protein and formation of DNA adducts (Valavanidis *et al.,* 2006). Very often, the lipid peroxidation which results from formation of highly reactive and unstable hydroperoxides in unsaturated lipids, is quantified through malondialdehyde level analysis (MDA), one of the final products from lipid collapse (Teixeira *et al.,* 2013).

Another method to evaluate ROS synthesis in marine organisms with efficient antioxidant capabilities, distinguishes itself due to several antioxidant enzymes which react as a whole to detoxicate ROS (Abele and Puntarulo, 2004). Hydrogen Peroxide (H_2O_2) is formed from the dismutation of the superoxide radical ($^{*}O^{-}_{2}$) by antioxidant enzymes. Mitochondria are important sources of $^{*}O_2^{-}$ and, as the presence of this free radical may cause serious damage, mitochondria are rich in SOD, which convert free radicals in H_2O_2. The antioxidant enzymes worth mentioning are: Superoxide Dismutase (SOD), which converts the superoxide radical ($^{*}O^{-}_{2}$) in hydrogen peroxide (H_2O_2); catalase (CAT), which contains hematin, and acts removing H_2O_2 to prevent its accumulation in cells and tissue, being metabolized into molecular oxygen and water; this enzyme is related with fat acids metabolism, and among with glutathione peroxidase (GPx) reacts against oxidative stress (figure 1.2.1) (Deisseroth and Dounce, 1970; Huggett *et al.,* 1992); glutathione S-transferase (GST) acts as a catalyst to

conjugate several electrophilic compounds with tripeptide glutathione, performing an important role in the protection against oxidative and peroxidative damage, lipids and DNA products (Henson *et al.,* 2001). GST and reduced glutathione (GSH) convert xenobiotics in on the conjugated; and glutathione reductase (GR) provides GSH to cells (Lesser, 2006; Teixeira *et al.,* 2013; Hebbel, 1986; Sies, 1993; Hermes-Lima and Zenteno-Savín, 2002).

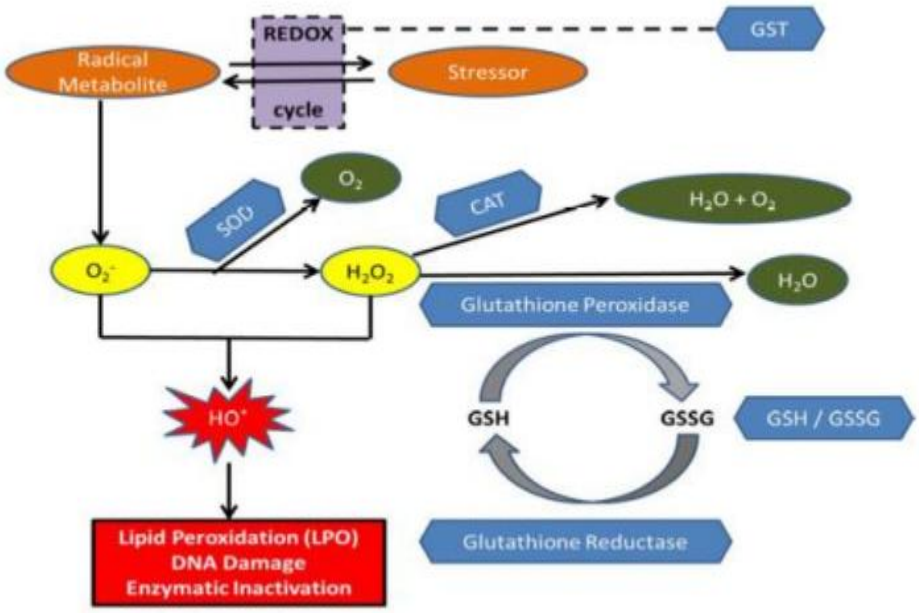

Figure 1.2.1 – Scheme oxidative stress induced by reactive oxygen species (ROS) (adapted from Ferreira, 2014)

Some authors, such as Hermes-Lima and Zenteno-Savín (2002) and Storey and Storey (2004), have observed a phenomenon of "preparation for oxidative stress state", in which some aquatic organisms developed a preparatory defense strategy, during a oxygen restrain period, and emersion, they activate antioxidant enzyme activity as a biological mechanism to minimize free radical damage after reoxygenation driven hypoxia. This way, when the amount of O_2 gets low, cellular hypoxia occurs and mitochondrial ROS is temporarily formed.

During reoxygenation, as oxygen concentration is drastically increased, ROS formation is also proportionally increased. As ROS levels arise, oxidative damage may occur and activate antioxidant defenses. This way ROS are considered signaling molecules involved in preparation for oxidative stress (figure 1.2.2).

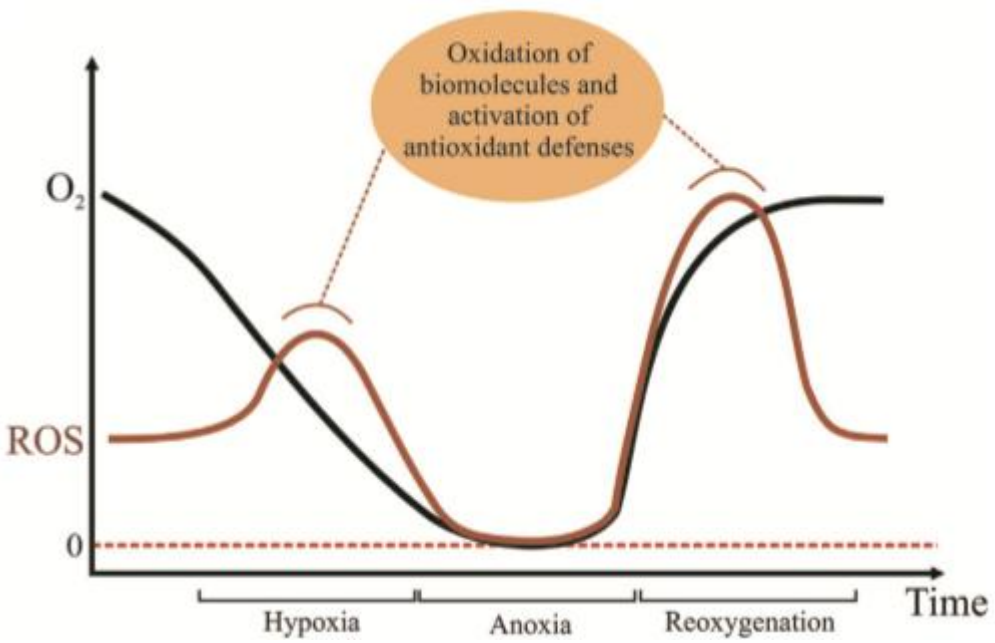

Figure 1.2.2 – Scheme demonstrating how ROS formation would behave during exposure to hypoxia/anoxia (adapted from Hermes-Lima *et al.*, 2015).

Thermal stress is also followed by oxidative stress in several sea mollusc species (Abele *et al.,* 1998, 2001). But, at the same time it hasn't been observed the existence of preparatory defense strategy mechanisms, to oxygen restrain periods in nudibranchs, and is not known if reoxygenation may cause cell damage to these organisms.

1.3- Slug species

1.3.1- Biology

Nudibranchs are marine gastropods which, during the course of their evolution, lost their shells. That loss was proportioned by the increasing biological, mechanical and physical defense strategies (Yonow, 2015). These are hermaphrodite organisms, characterized by the presence of granular prostate. This gametophyte has two cavities, one of them connects with seminal receptacle while the other connects with female sex glandule. Opposite sex glandules are separated by digestive glandule (Valdés *et al.,* 1996). The great majority of nudibranchs show a lively coloration of their mantle, with patterns that allow us to distinguish some species. These are also bilaterally symmetrical, usually carrying a pair of rhinophores (Yonow, 2015). Their gills are disposed in circles, being later shut tight by their anus. Rhinophores show a cylindrical lamellated stem, their mouth tentacles are very small or nonexistent (Valdés *et al.,*

1996). Nudibranch group and taxonomic classification has been permanently targeted throughout the years (Yonow, 2015). The Dendrodoris species, for instance, presents a simple, spicule less body. These spicules usually occur during minutes in isolated cases. The mantle border is delicate, usually wide and undulated, and presenting stretch markings in some species. This way, radule absence and variable color, makes *Dendrodoris sp.* Species to be highly subjective (Valdés *et al.,* 1996).

In 1996, Valdés described 23 species of *Dendrodoris sp.* existing in the Atlantic Ocean, but it was very hard to distinguish from each other mainly due to the many similarities in their external morphology of this kind. This way, more detailed observation of their internal anatomy is sometimes necessary (Hirose *et al.,* 2015).

The model organisms in this study are *D. grandiflora* and *D. herytra*, whose biology and relevant characteristics, distribution, habitat, feeding and relevant behaviour are described in more detail.

1.3.2- *Dendrodoris grandiflora* Vs *D. herytra*

1.3.2.1- *D.grandiflora*

D. grandiflora has a body with approximately 7cm length, and its color varies a lot. The pattern color in Mediterranean species is gray, cream, greenish, brown or reddish, accompanied with black or brown spots; in northern Africa and Canarian Islands, they are usually red or orange; in Portugal, the majority shows yellow color, with black or brown spots. These are irregular spots, with different sizes which sometimes may cover the whole mantle. In their belly, these organisms have the same color than the mantle, but without spots. Rhinophores and branches are usually the same color than the mantle, but apex pigmentation is white. Juvenile are uniformly reddish. And its mantle borders very striped and wide when compared to their feet (Valdés, 2000) (figure 1.3.1).

1.3.2.2- *D.herytra*

D. herytra has a body with approximately 4 to 5cm length, its coloration is a reddish uniform, some are orange yellow, and sometimes pale green. Their rhinophores and gills are the same color of their body, with an white rhinophores apex and gills. Juvenile are all uniformly reddish, but may develop soft wrinkles in some specimen. Gills are relatively smaller than *D. grandiflora* (Afonso, 2006) (figure 1.3.1).

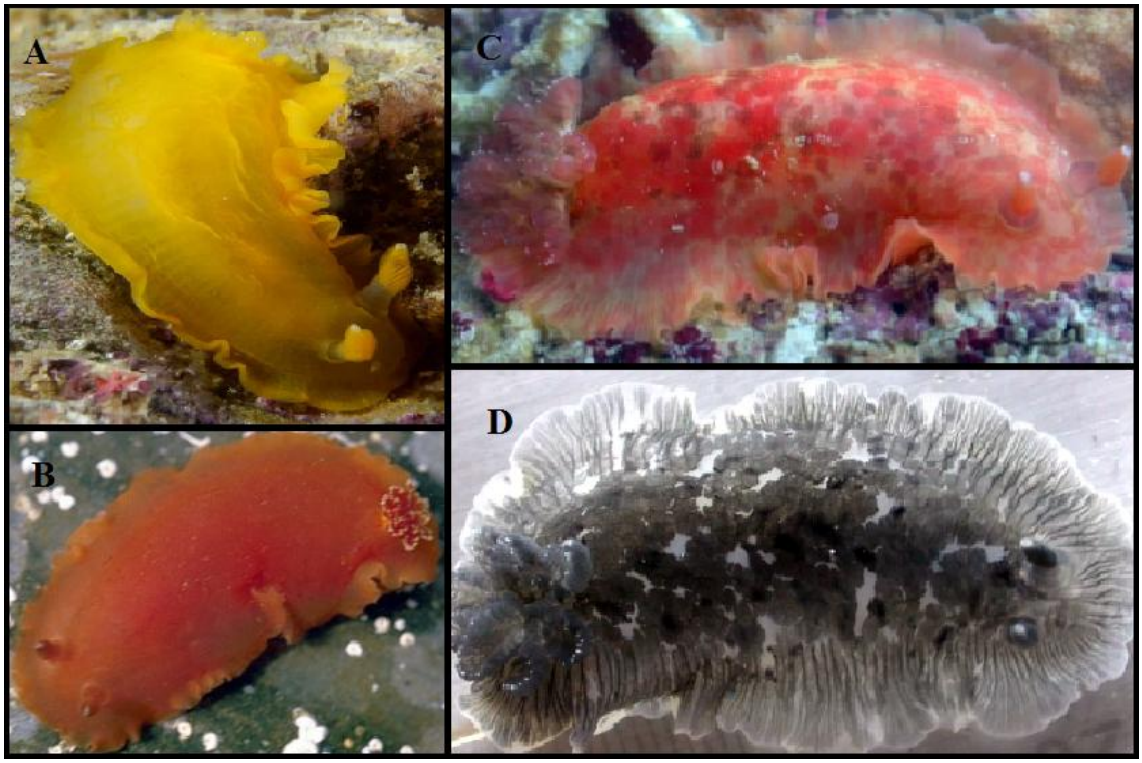

Figure 1.3.1 - A and **B** – *D. herytra*; **C** and **D** – *D. grandiflora* (photos adapted from Afonso, 2006 and Valdés, 2000, respectively).

1.3.3- Feeding

The suborder Dendronotacea presents a diverse diet are able to predate many organisms, but rather prey on benthic cnidarians (Hoover *et al.,* 2012), corals, sponges and tunicates (Crane, 1972).

1.3.4- Distribution

Nudibranchs present a very wide distribution, being resident of many ecological niches, inhabiting from tropical to arctic ecosystems (Dionísio *et al.,* 2013). These are primarily subtidal species, although can be found close to medium sea level or even above (Hoover *et al.,* 2012).

1.3.4.1- Distribution of *D. grandiflora*

This species inhabits the Mediterranean Sea, from Turkey and Israel, up to the straight of Gibraltar. In the Atlantic Ocean it has been observed in Portugal, Canarian Islands, and the coast of Sahara (Valdés *et al.,* 1996) (figure 4).

1.3.4.2- Distribution of *D. herytra*

This species has only been observed in the northeastern Atlantic Ocean, from the Gulf of Biscay to the Strait of Gibraltar, and from the coast of Morocco to the coast of Mauritania. It has also been observed in the Azores and Madeira Islands, but the great majority was in northern Spain, and south of the Canarias Islands (Valdés *et al.,* 1996) (figure 4).

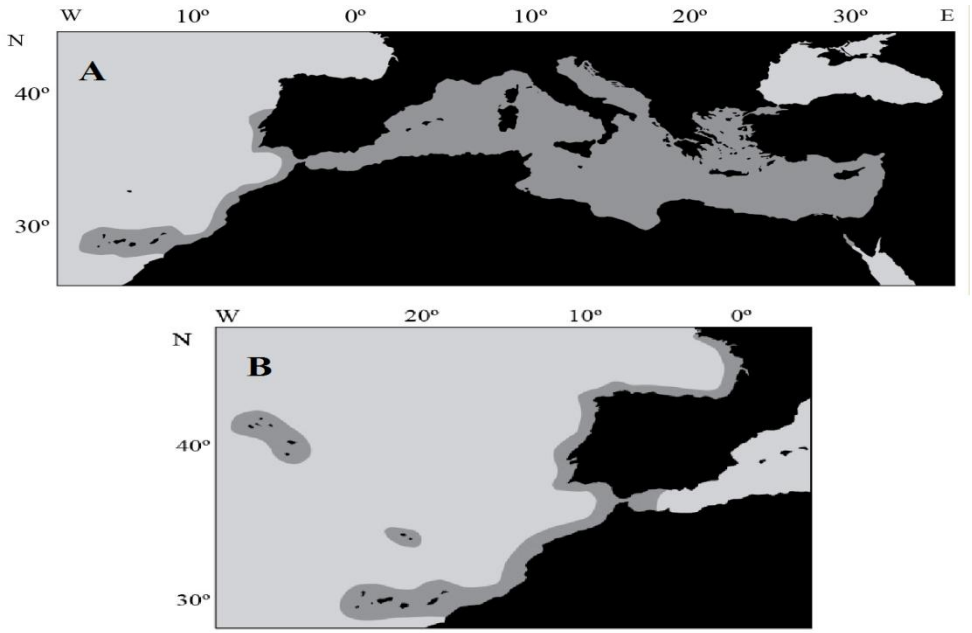

Figure 1.3.4.1 – Geographic distribution of species *D. grandiflora* (A) and *D. herytra* (B)

1.3.5- Habitat

These organisms live in irregular rocky substrates usually related with coral, sponges and strong undulated ocean currents (Behrens and Valdés, 2004). The two species in this study may be observed up to 25m depth, although *D. herytra* is usually found in shallow water depths, hidden between rocks and cracks (Calado and Silva, 2012).

1.3.6- Behaviour ecology

Several studies have shown that the great majority of nudibranch populations suffer from seasonal flotation due to physical (temperature and wave action) and/or biological events (competition and predation) (Costello, 1938; Harris, 1973; Todd, 1981, 1983).

In 1938, Costello tried to explain the events in population density with a theory about migrating organisms from subtidal to upper intertidal areas, as a part of reproduction cycle, so they could copulate and lay egg masses. Although these are hermaphrodite creatures, they look for a partner to mate with.

This way, these organisms would become exposed to quite stressful environmental events for their survival, such as the amount of available oxygen (Claverie and Kamenos, 2008).

In an intertidal environment is essential that the animal can tolerate several levels of air exposure, and may repress respiratory rate in long periods of exposure, or be able to assimilate atmospheric oxygen (Ghiretti, 1966). So this makes it crucial that anatomical and behavioral changes take place, allowing oxygen to be spread throughout their entire body in alternative structures, when the usual ones aren't fully functional (Potts, 1983).

In 1970, Potts suggest that nudibranchs complement oxygen consumption through skin breathing, being their mantle quite irrigated, possessing several blood vessels which make gaseous trades with their environment. In 1983, Potts observed that in Doridacea order nudibranchs, when immerse, the gills expand and breathing intensifies, hitting their maximum level of oxygen consumption. The importance of relative gills depends on the size of the organism, and it is known that smaller sized individuals possess reduced gills. This way, it´s important that mollusc groups which populate intertidal areas do not totally rely on their gills for survival.

1.4- Objectives

This dissertation aimed to investigate the physiological mechanisms of two species of nudibranchs (*Dendrodoris grandiflora* and *D. herytra)* that undergo daily emersion during low tides. More specifically, this study was focused on the quantification of: i) HSP70 expression ii) lipid oxidation, iii) oxidative stress (activity of antioxidant enzymes), in order to understand if these mollusk species display preparatory defense strategies to, air exposure (emersion) and reoxygenation (immersion).

2- Materials and methods

2.1-Sampling and exposure of nudibranchs the emergence and immersion

52 *D. grandiflora* and 27 *D. herytra* have been captured on an oyster bank with a total area comprising 1050m^2, located in Mitrena, Sado's Estuary National Reserve, west Portugal (38.474857ºN, -8.775255ºW; figure 5). The sampling was conducted only when this bank was found completely emersed. Paths were made so that all area was covered in this study, in tide periods lower than 0.50 m covering the time period from April to June 2014 and 2015. In the sampling site the temperature, salinity and pH were measured, obtaining the following medium values of 21ºC, 34 and 8.2, respectively.

The captured organisms were transported to the lab and acclimated for 24 hours, prior the beginning of the experiment, in a refrigerated closed system (22ºC, 33 salinity and approximately 100% dissolved oxygen), with UV-sterilized and filtered water (in a very close environment to their original habitat). 5 random individuals were later sampled from each species, conveniently identified them and then frozen in -80ºC. These organisms were considered control group. Next, the remaining ones were placed inside a container (in the open air, in a natural radiation condition) with previously picked subtract from Mitrena, so to mimic the oyster bank where they were picked. Then a 2-hour emersion and 2-hour immersion tide period were simulated, and during this period 6 *D. grandiflora* and 4 *D. herytra* specimen were sampled and individually frozen with 30 minute gaps. The last collected organism was after 2 emersion hours, because this was considered representative of the maximum amount of air exposure time in that place.

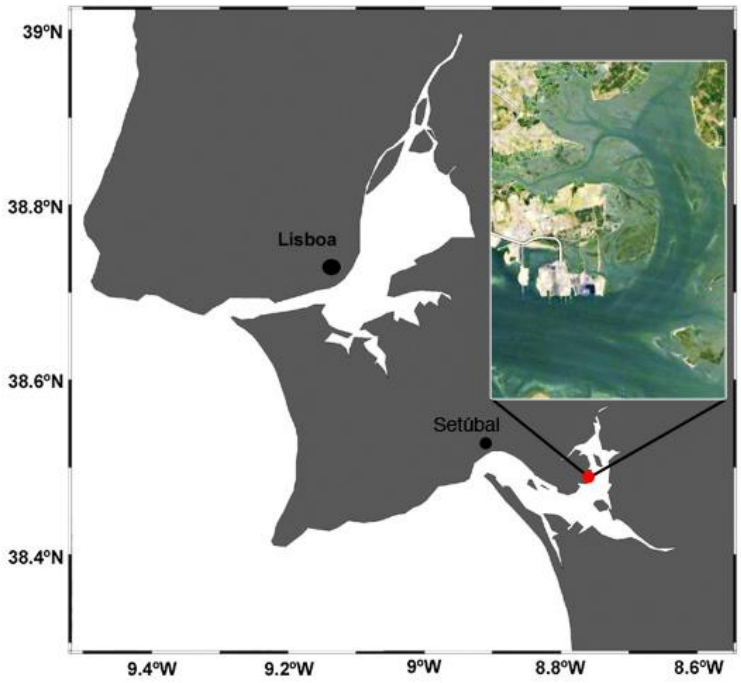

Figure 2.1.1 – Map of the sampling area, Mitrena, located in the Sado's Estuary, Portugal.

2.2- Analysis

2.2.1. Preparation of tissue extracts

The tissue for homogenization was selected from a cut made along the organism width, close to the head and weighing approximately 210mg. Homogenization was prepared with a tampon solution, phosphate buffered saline solution (PBS; pH 7.4) (0,14M NaCl, 2,7 mM $Na2PO_4$, 1,47 mM KH_2PO_4). The samples were homogenizated with approximately 715 L of the homogenization buffer, phosphate buffered saline solution (PBS, pH 7.3): 0.14M NaCl, 2.7 mM KCl, 8.1 mM Na2HP04, 1.47 mM KH2P04) by using a glass/PTFE Potter Elvehjem tissue grinder (Kartell, Italy). All homogenates were then centrifuged (20 min at 14,000×g at 4ºC) and enzyme activities and protein expression were measured in the supernatant fraction. All enzyme assays were tested with commercial enzymes obtained from Sigma (Missouri, USA), and each sample (colony) was run in triplicate (technical replicates).

2.2.2- Heat shock protein (HSP70)

HSP70/HSC70 content was assessed by Enzyme-Linked Immunoabsorbent Assay (ELISA) adapting a protocol from Njemini et al. (2005). Briefly, 10 μL of the

homogenate's supernatant was diluted in 250 µl of PBS (1x), and 50 µL of the diluted sample was added to a 96-well microplates (Nunc-Roskilde, Denmark) and allowed to incubate overnight at 4°C. On the next day the microplates were washed (3X) in 0.05% PBS-Tween-20. One hundred microliters of blocking solution (1% bovine serum albumin [BSA] Sigma-Aldrich, USA) was added to each well and left to incubate at room temperature for 2 h. After washing of the 96-well plates, 50 µL of 5 µg/mL primary antibody (anti-HSP70/HSC70, Acris, USA), detecting 72 and 73 kDa proteins corresponding to the molecular mass of inducible and constitutive isoforms, respectively, was added to each well and then incubated at 37ºC for 90 min. The non-linked antibody was removed by washing the microplates again, which were then incubated for 90 min at 37 ºC with 50 µL of 1 µg/mL of the secondary antibody, anti-mouse IgC, Fab specific, alkaline phosphatase conjugate (Sigma-Aldrich, USA). After three additional washes, 100 µL of substrate (SIGMA FASTTM p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to each well and incubated 10-30 min at room temperature. Fifty microliters of stop solution (3N NaOH) was added to each well, and the absorbance was then read at 405 nm in a 96-well microplate reader. The amount of HSP70/HSC70 in samples was calculated from a curve of absorbance based on serial dilutions of purified HSP70 active protein (Acris, USA) to give a range from 0 to 2000 ng/mL. Results were expressed in relation to the sample wet weight (ng HSP70/HSC70 mg−1 ww).

2.3.3. Glutathione S-Transferase (GST)

GST activity was determined according to the procedure described by Habig et al. (1974) and optimized for 96 well microplate. This assay uses 1-Chloro-2,4-dinitrobenzene (CDNB) as substrate and, upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance. Therefore, the enzyme activity was determined spectrophotometrically by measuring the formation of the conjugate of glutathione (GSH) and l-chloro-2,4-dinitrobenzene (CDNB). The assay contained 200mM L-glutathione (reduced), Dulbecco's PBS and 100mM 1-chloro-2,4-dinitrobenzene (CDNB) solution. Equine liver GST (Sigma-Aldrich, Germany) was used as positive control to validate the assay. Then, to perform the assay, 180 µL of substrate solution were added to 20 µL of GST standard or sample in each well of a 96-well microplate (Nunc-Roskilde, Denmark) and the absorbance at 340 nm was recorded

every minute for 6 min, using a plate reader (BioRad, California, USA). The increase in absorbance per minute was estimated and the reaction rate at 340 nm was determined using the CDNB extinction coefficient of 0.0053 µM-1cm-1 as follows:

GST activity=(ΔA_340/min)/0,0053×(Total volume)/(Sample volume) x dilution factor
The results are expressed in relation to wet weight of the sample (pmol min−1 mg−1 ww).

2.3.4. Catalase (CAT)

The assay for the determination of catalase activity (EC 1.11.1.6) was based on Aebi (1984). In this assay, CAT activity is usually assessed by measuring the rate of removal of H2O2. Consequently, the reaction can be followed by a decrease in absorbance as the H2O2 is turn into oxygen and water. At the end of the assay, H2O2 is consumed and CAT is inactivated. The total reaction volume of 3 mL was composed of 50 mM potassium phosphate buffer (pH 7.0), 12.1 mM H2O2 as a substrate, and the reaction was started by the addition of the sample into quartz cuvettes with a path length of 10mm. The consumption of peroxide (extinction coeff. 0.04 mM cm−1) was monitored using a Helios spectrophotometer (Unicam, UK) at 240 nm and 25°C at 15 s intervals across a 180 s incubation period. Standard catalase activity was measured using a bovine catalase solution (1523.6 U mL-1) as a positive control for validation of the assay.

Catalase activity was calculated using the following equation:

(ΔA_240/min)/0,04 ×(Total volume)/(Sample volume)

The results are expressed in relation to the wet weight of the sample (pmol min−1 mg−1 ww).

2.3.5. Superoxide dismutase (SOD)

SOD is responsible for catalyzing the breakdown of superoxide radicals, providing a first defense against O2 toxicity. The SOD enzyme assay followed the the nitroblue tetrazolium (NBT) method adapted from Sun et al. (1988). In this method, superoxide radicals (·O2) are generated by the reaction of xanthine with xanthine-oxidase (XOD), and reduce NBT to formazan. SOD will compete with NBT for the dismutation of·O2 into peroxide (H2O2) and elemental oxygen (O2). The percent inhibition of NBT reduction is used as a measure of SOD activity. Then, SOD activity was determined

spectrophotometrically in the supernatant at 25 °C (BIO-RAD, Benchmark, USA) at 550 nm. The adapted assay (25 °C) contained 50mM potassium phosphate buffer (pH 7.8), 3mM EDTA, 3mM xantine solution, 0.75mM NBT, 100mU XOD. SOD from bovine erythrocytes (Sigma-Aldrich, Germany) was used to build a standard curve and as positive control (1 U/□L SOD Enzyme solution). The results of this enzymatic assay are given in units of SOD activity per milligram of wet weight of the sample (U mg-1 ww). Since one unit of SOD inhibits the rate of increase in absorbance at 550 nm by 50 % the percent inhibition of the samples correlates with SOD activity using a SOD standard curve.

2.3.6. Determination of malondialdehyde concentration

Lipid peroxidation was determined by the quantification of a specific end-product of the oxidative degradation process of lipids, malondialdehyde (MDA). Thiobarbituric acid reactive substances (TBARS) assay (Uchiyama and Mihara, 1978) was used, in which thiobarbituric acid reacts with MDA to yield a fluorescent product that was detected spectrophotometrically at 532 nm. Homogenates were treated with 8.1 % sodium dodecyl sulfate, 20% trichloroacetic acid (pH 3.5), thiobarbituric acid and a 15: 1 (v/v) mixture of n-butanol and pyridine (Sigma-Aldrich, Germany) (Correia et al., 2003). MDA concentrations were calculated with the computer program Microplate Manager 4.0 (BIO-RAD, USA) based on an eight-point calibration curve (0-0.3 μM TBARS) using MDA bis (dimethyl acetal; Merck; Switzerland). Results were expressed in relation to the sample wet weight (pmol mg−1 ww).

2.2.7- Total protein content

The total protein of all biomarker measurements was quantified through Bradford method (Bradford, 1976), adapted to 96 well plates.

2.3- Statistical analysis

An analysis of variance (ANOVA) was conducted to verify if HSP70/HSC70, MDA, CAT, SOD and GST expression temporal variations were significant. When needed, data was transformed to satisfy normal distribution and homoscedascity requirements. T-test was used to check for differences in variations between both species being studied in each time interval. Normality and homogeneity was analysed, in both tests, through Kolmogorov-Smimov & Lilliefors test for normality and Levene's

test for homoscedascity, respectively. Statistic analyses were conducted using STATISTICA version 12.0 (StatSoft Inc) software, with a 0.05 significant level.

3- Results

Regarding *D. grandiflora*, a significant HSP70 expression increase was verified during emersion, with a maximum value (10.32 ng^{-1}mg total protein) after 60 minutes of air exposure. Then, after a few hours of reoxygenation, HSP70 levels gradually decreased. But, in comparison to *D. grandiflora*, *D. herytra* HSP70 expression levels during immersion periods did not vary significantly, with the exception of a maximum value of 4.87 ng^{-1}mg total protein (figure 3.1). MDA expressions during emersion periods are very unstable. At 30 minutes, *D. grandiflora* species presents a lipid peroxidation expression inhibition, from 2.33 to 1.64 $nmol^{-1}mg^{-1}$total protein, followed by a 2.62 $nmol^{-1}mg^{-1}$total protein increase. At 90 minutes, another enzyme inhibition to 1.65, followed by a increase to 2.64 $nmol^{-1}mg^{-1}$total protein. After the immersion, MDA levels tend to decrease to 1.28 $nmol^{-1}mg^{-1}$total protein, significantly increasing at 240 minutes. To *D. herytra* species, the same tendency during immersion have been observed (figure 3.2).

SOD varied between 1.70 to 1.97% inhibition mg^{-1}total protein at 60 minutes of emersion, gradually decreasing to 1.36% inhibition mg^{-1} total protein at 180 minutes, increasing again after 2 hours of reoxygenation, at 240 minutes. In the other hand, *D. herytra* species during reoxygenation period decreased from 1.57 to 1.26% inhibition mg^{-1}total protein at 240 minutes (figure 3.3). GST tended to increase during emersion, with the exception of a breach at 90 minutes when the lowest value of 36.46 $nmol^{-1}min^{-1}mg^{-1}$total protein appeared. After reoxygenation was verified the opposite tendency, in which values tend to decrease even after a small recovery at 180 minutes. The same decrease tendency occurred during *D. herytra's* immersion period (figure 3.4). With *D. grandiflora* species, CAT presents very unstable values during emersion, in the opposite way to MDA, showing a increase from 3.92 to 9.68 $nmol^{-1}min^{-1}mg^{-1}$total protein at 30 minutes, followed by a decrease to 5.65 $nmol^{-1}min^{-1}mg^{-1}$total protein at 60 minutes, then rising again, at 90 minutes, up to 8.04 $nmol^{-1}min^{-1}mg^{-1}$total protein and decreasing to 3.86 $nmol^{-1}min^{-1}mg^{-1}$total protein at 120 minutes. In the reoxygenation interface was verified a slight increase to 5.88 $nmol^{-1}min^{-1}mg^{-1}$total protein, remaining stable during immersion except at 210 minutes, when a minimum expression occurred, and then recovered again. During the immersion period

D. herytra species shown tendency to a very smooth increase, with relatively low and stable values (ranging from 2.25 to 3.13 $nmol^{-1}$ $min^{-1}mg^{-1}$total protein) (figure 3.5).

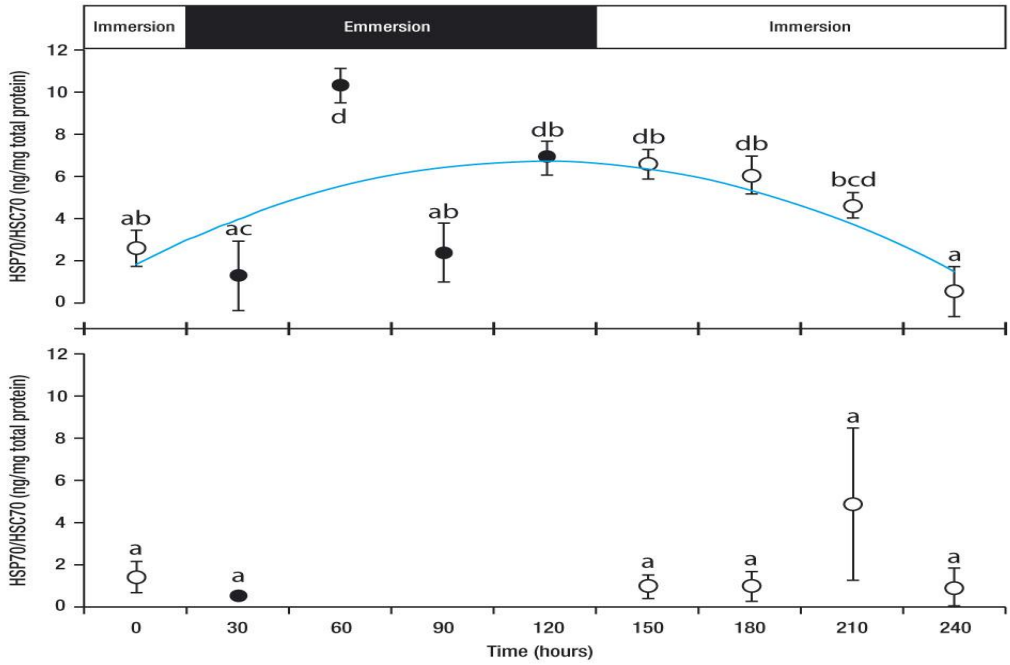

Figure 3.1 - Changes in heat shock protein expression (HSP70/HSC70 ng^{-1}mg total protein) during air exposure (emersion) and reoxygenation (immersion) in the *D.grandiflora* and *D.herytra*. Values represent means ± SD (n=5 species). Different letters significant differences between time periods ($p<0.05$).

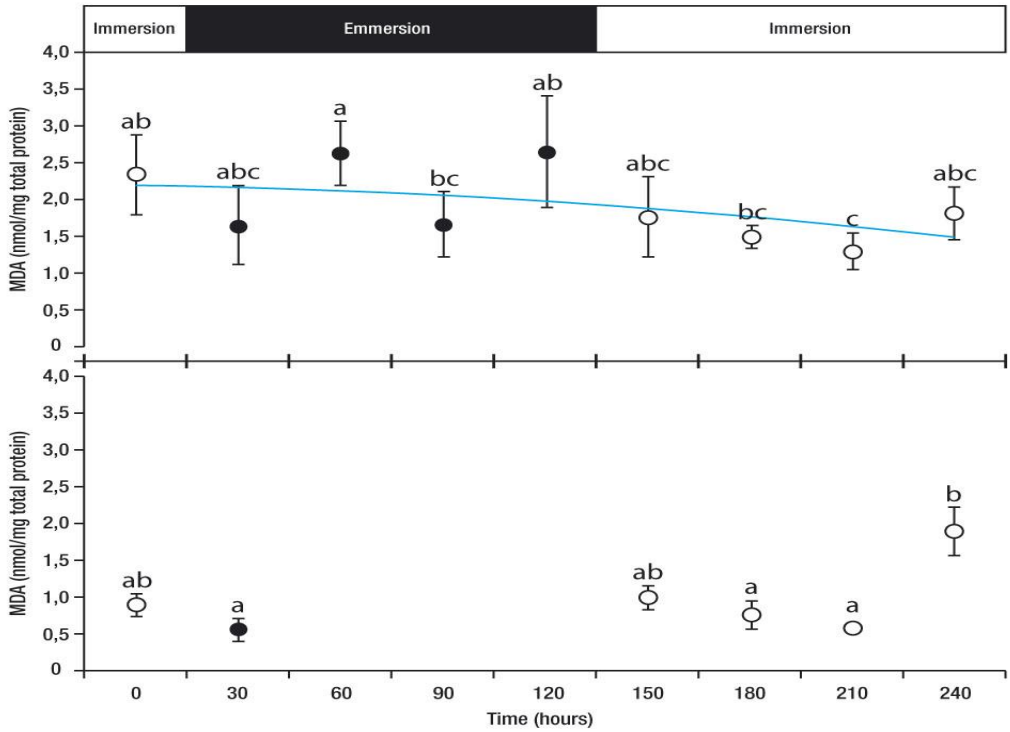

Figure 3.2- Changes in the malondialdehyde (MDA $nmol^{-1}mg^{-1}$total protein) concentrations during air exposure (emersion) and reoxygenation (immersion) in the *D.grandiflora* and *D.herytra*. Values represent means ± SD (n=5 species). Different letters significant differences between time periods ($p<0.05$).

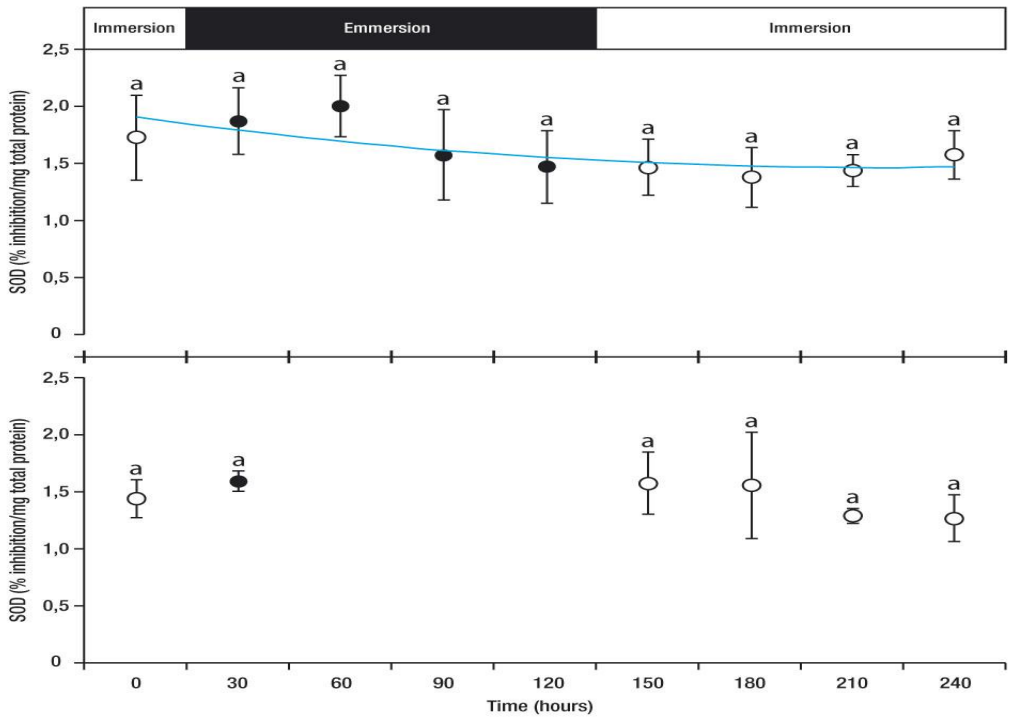

Figure 3.3- Changes in the superoxide-dismutase (SOD % inhibition mg^{-1}total protein) activity during air exposure (emersion) and reoxygenation (immersion) in the *D.grandiflora* and *D.herytra*. Values represent means ± SD (n=5 species). Different letters significant differences between time periods ($p<0.05$).

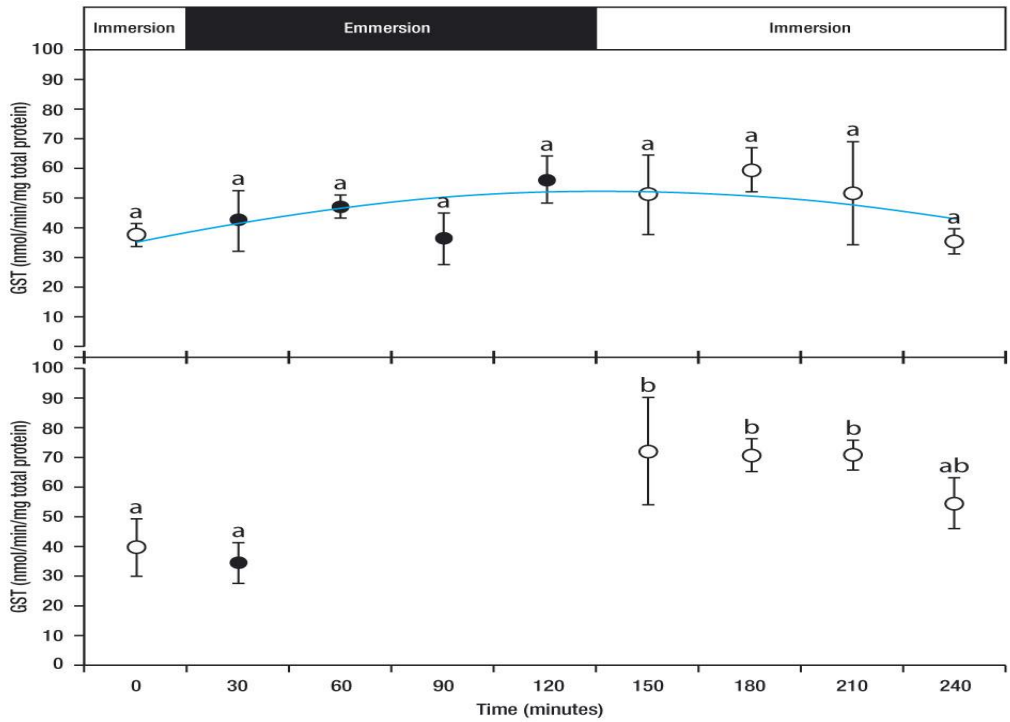

Figure 3.4- Changes in the glutathione-S-transferase (GST $nmol^{-1}min^{-1}mg^{-1}$total protein) activity during air exposure (emersion) and reoxygenation (immersion) in the *D.grandiflora* and *D.herytra*. Values represent means ± SD (n=5 species). Different letters significant differences between time periods ($p<0.05$).

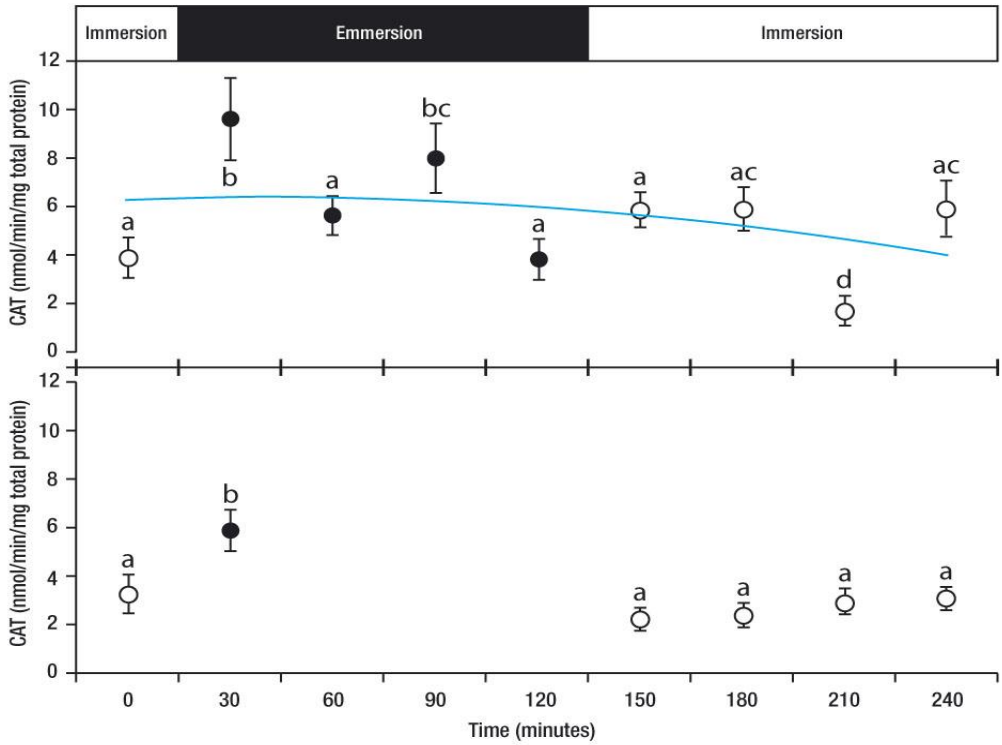

Figure 3.5- Changes in the catalase (CAT $nmol^{-1}min^{-1}mg^{-1}$total protein) activity during air exposure (emersion) and reoxygenation (immersion) in the *D.grandiflora* and *D.herytra*. Values represent means ± SD (n=5 species). Different letters significant differences between time periods ($p<0.05$).

4- Discussion

Intertidal organisms are subjected to tidal cyclical fluctuations, as well as environmental parameters such as air exposure, ultraviolet radiation, salinity and hydrodynamic forces (Aguilera and Rautenberger, 2010). Air exposure is the abiotic factor who stresses the most these organisms, gradually causing internal tissue hypoxia, leads to cellular protein damage or death (Teixeira *et al*., 2013).

Hypoxia causes the loss of oxidative phosphorylation and mitochondria ATP production, stopping the cells from using their primary mean of energy supply. The absence of energy will then lead to a series of metabolic and morphologic changes in these cells. Such changes make them unable to survive prolonged periods of O_2 deprivation, may cause membrane damage and the loss of cellular ion homeostasis in vital organs and tissues that cannot be reversed (Hochanchka and Somero, 2002).

In low oxygen concentration conditions, the cells continue to require the same concentration to function, causing an energy balance deficit. This energy deficit may activate the anaerobic ATP supply pathways. However, this mechanism can't produce

enough energy to the biological processes in the cells, because the fermentable substrate is rapidly depleting, and at the same time there is accumulation of harmful end products. The complexity of biochemical and physiological mechanisms allow organisms to survive under these extreme conditions, and to be able to recover from hypoxia caused by exposure at low tide (Hochachka *et al.,* 1996).

Therefore, according to the collected data, we can state that one of the chances to survival under hypoxic conditions is to be inherently able to dysregulate their cellular metabolic rate to new hypometabolic steady states. This ability to suppress the metabolism can balance ATP demand and supply pathways (as been observed with jumbo squids thriving in oxygen minimum zones (Rosa and Seiel, 2008, 2010) and with coral in air exposure (Teixeira *et al.,* 2013).

A reduction in antioxidant enzymes was to be expected, once ROS production would probably be reduced during periods of low tissue oxygenation. However, the contrary has been observed, as air exposed antioxidant enzymes exhibited higher SOD activity.

Hermes-Lima *et al.* (1998) observed that during estivation, in the case of the land snail, there was an increase in the antioxidant defenses, suggesting that this response was a preparative mechanism against oxidative stress during arousal. Similar to this, many other stress tolerant animals would display coordinated changes in their antioxidant defenses that allow them to deal with oxidative stress that occurs as part of the natural cycles of stress and recovery that change oxygen levels in the tissues.

In this experiment, nudibranch exposed to air under oxidative stress behaved with irregular enzymatic concentration. Notoriously displaying an integrated stress response that involves the antioxidant defense system and heat shock expression (HSR).

The synthetization of these enzymatic proteins is very important to repair, refolding and elimination of damaged proteins (Sokolova *et al.,* 2011), and their expression varies in a period of time which approximates the duration of many tidal cycles (Dong *et al.,* 2008; Tomanek, 2010). During the emersion phase, which is associated with a gradual rise in hypoxia, there was a significant increase in GST, SOD and HSP activities, whereas CAT and MDA activity remained stable. This increase of GST, SOD and HSP activity is considered as an anticipatory protective response to oxidative stress of the reoxygenation event (Hermes-Lima and Zenteno-Savín, 2002). The increased levels of HSC70/HSP70 observer during emersion represent a strategy of preparatory defense to prevent the damage caused by the reoxygenation process. During

reoxygenation stage (namely after 30 min) was notorious a significant enzyme production with tends to attenuate throughout the recovery period. The GST and HSP levels are significant during the emersion phase, demonstrating that nudibranchs are in oxidative stress when exposed to air. This preparation is considered the most important key in the protection against post-hypoxic free radical damage (Freire *et al.,* 2011). On the other hand, these organisms may choose physical strategies like behavioral mechanisms, such as shell gapping, may enhance their survival at low tides, where small movements in the shell can help them maintaining their aerobic metabolism and removing of anaerobic waste products (Demers and Guderley, 1994).

ROS increase during hypoxia/reoxygenation may happen due to oxidated cellular components or reactions with produce other oxidant species, such as hydroperoxide lipids, increasing biomarker levels, transcription factors, increasing the expression of antioxidant defenses, or by activating the pathways which cause post translational modifications in antioxidant enzymes, in a way to improve the antioxidant system. After some time the ROS formation peak will eventually decrease, as well as its effects. However, lipidic peroxidation electrophile products may extend the antioxidant expression, maintaining POS response in hypoxia in the long term. According to Hermes-Lima *et al.,* (2015) (figure 5.1), when an organism is exposed to an hypoxia environment, ROS formation increases causing the biomolecules to oxidize (such as GSH, lipidic membrane, etc), Electrophilic product formation due to lipid peroxidation may activate transcription factors Nrf2 and contribute to POS response to long hypoxia exposure. Activate reduction sensitive transcription factors, increasing antioxidant enzyme expression. And still, the covalent modification of antioxidant protein may increase antioxidant enzyme activity, contributing to POS response.

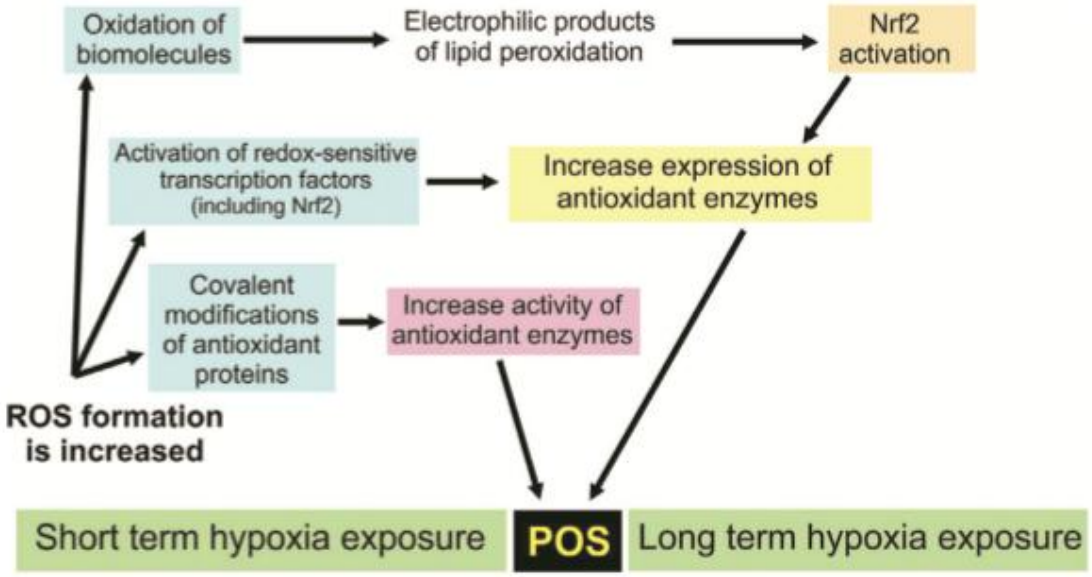

Figure 5.1 – Illustrative scheme of Preparative Oxidative Stress (POS) mechanisms (adapted from Hermes-Lima *et al.*, 2015).

Oxidation is a fundamental part of aerobic life and our metabolism and so, free radicals are naturally produced by some biological disfunction. Many animal species can survive for long periods of time without oxygen, including several vertebrate and invertebrate ones. One well known mechanism used in survival at low oxygen levels is the accurate depression of metabolic rate during these periods of oxygen deprivation associated with low levels of ATP production. This ability to slow down the energy consumption mechanism is a key strategy for survival, including the reduction of enzyme activity. Mitochondria is the main source of excessive ROS formation during reoxygenation. Yet, it is worth noting that, currently, there is no specific evidence of mitochondrial ROS formation during estivation or stress due to low oxygen levels, i.e., in the measurement of the ROS levels are used, as an experiment, chemical indicators that allow us to check the ROS formation during the anoxia. (Milton *et al.,* 2007; Rivera-Ingraham *et al.,* 2013), but with these we can't state exactly when the increase of ROS occurs, and consequently the activation of the antioxidant defenses. These limitations were also encountered by other authors in simulant situations but in different species, in particular cnidarians (corals), annelids (polychaetes), tardigrades, molluscs (bivalves and gastropods), arthropods (crustaceans and insects), and vertebrates (fish, amphibians and reptiles) (Hermes-Lima *et al.,* 2015).

So, as it seems, ROS formed at an early stage of estivation or low oxygen stress could activate endogenous antioxidants and inflict oxidative damage to biomolecules in animals that tolerate anoxia and hypoxia.

5- References

Abele, D. and Puntarulo, S. (2004). Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 138(4), 405-415.

Abele, D., Burlando, B., Viarengo, A. and Pörtner, O. (1998). Exposure to elevated temperatures and hydrogen peroxide elicits oxidative stress and antioxidant response in the Antarctic intertidal limpet *Nacella concinna*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 120(2), 425–435.

Abele, D., Tesch, C., Wencke, P. and Pörtner, O. (2001). How do oxidative stress parameters relate to thermal tolerance in the Antarctic bivalve Yoldia eightsi? Antarctic Science 13(2), 111–118.

Aebi, H., (1984). Catalase. In: Packer, L. (Ed.), Methods in Enzymology. Academic Press, Orlando 105, 121 – 126.

Afonso, L., (2006). *Dendrodoris grandiflora* from southern Portugal. *[Message in] Sea Slug Forum.* Australian Museum, Sydney.

Aguilera, J., Rautenberger, R., (2010). Oxidative stress tolerance strategies of algae living in the intertidal. in: Abele, D., Zenteno-Savín, T., Vazquez-Medina, J. (Eds.), Oxidative Stress in Aquatic Ecosystems. John Wiley & Sons, Ltd, Chichester, UK. 58–71

Almeida, E., Bainy, A. (2006). Effects of Aerial Exposure on Antioxidant Defenses in the Brown Mussel Perna perna. Biological and applied sciences 49, 225-229.

Behrens, W., Valdés, Á. (2004). A New Species of Dendrodoris (Mollusca: Nudigills: Dendrodorididae) from the Pacific Coast of North America. Proceedings of the California Academy of Sciences 55, 408–413.

Bradford, M., (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principie of protein-dye binding. Annals of Biochemestry.72, 248-254.

Calado, G., Silva, P. (2012). Lesmas do mar do Algarve - Guia de moluscos opistobrânquios da costa sul de Portugal. Lisboa - Portugal, Subnauta.

Ciavarra, P., Goldman, C., Wen, K., Tedeschi, B., Castora, J. (1994). Heat stress induces hsc70/nuclear topoisomerase I complex formation in vivo: evidence for hsc70-mediated, ATP-independent reactivation in vitro. Proceedings of the National Academy of Sciences 91(5), 1751-1755.

Claverie, T., Kamenos, A. (2008). Spawning aggregations and mass movements in subtidal *Onchidoris bilamellata* (Mollusca: Opisthogills). Journal of the Marine Biological Association of the United Kingdom 88(01), 157-159.

Correia, D., Costa, H., Luis, J., Livingstone, R. (2003). Age-related changes in antioxidant enzyme activities, fatty acid composition, and lipid peroxidation in whole body *Gammarus locusta* (Crustacea: Amphipoda). Journal of Experimental Marine Biology and Ecology. 289(1), 83–101

Costello, P. (1938). Notes on the breeding habits of the nudibranchs of Monterey bay and vicinity. Journal of Morphology 63(2), 319-343.

Crane, S. (1972). Population Ecology of the Nudibranch *Archidoris montereyensis.* Simon Fraser University, Department of Biological Sciences.

Demers, A., Guderley, H., (1994) Acclimatization to intertidal conditions modifies the physiological responses to prolonged air exposure in *Mytilus edulis*. Marine biology. 118, 115-122.

Diesseroth, A., Dounce, L., (1970). Catalase: physical and chemical properties, mechanisms of catalysis and phisiologycal role. Physiological Reviews. 50, 319-375.

Dionísio, G., Rosa, R., Leal, C., Cruz, S., Brandão, C., Calado, G., Serôdio, J., Calado, R. (2013). Beauties and beasts: A portrait of sea slugs aquaculture. Aquaculture 408–409.

Dong, Y., Miller, L., Sanders, J., Somero, G. (2008). Heat-Shock Protein 70 (Hsp70) Expression in Four Limpets of the Genus Lottia: Interspecific Variation in Constitutive and Inducible Synthesis Correlates With in situ Exposure to Heat Stress. The Biological Bulletin. 215, 173-181

Ferreira, C., (2014). O efeito de químicos em isópodes: uma avaliação multi-organizacional. Tese de Douturamento, Universidade de Aveiro.

Freire, C., Welker, A., Storey, J., Storey, K., Hermes-Lima, M. (2011). Oxidative stress in estuarine and intertidal environments (temperate and tropical). In:

Abele, D., Vázquez-Medina, P., Zenteno-Savín, T. (Eds.), Oxidative stress in aquatic ecosystems. Jonh Wiley & Sons. Chichester. 41-57.

Gestel, M., Brummelen, C. (1996). Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. Ecotoxicology. 5, 217-225.

Ghiretti, F. (1996). Respiration. In Physiology of Mollusca. (Eds). Wilbur, K. and Yonge, Part C. 2, 175-208. Academic Press.

Gracey, A., Chaney, M., Boomhower, J., Tyburczy, W., Connor, K., Somero, N. (2008). Rhythms of gene expression in a fluctuating intertidal environment. Current Biology. 18, 1501–1507.

Habig, W., Pabst, M., Jakoby, W. (1974). Glutathione S-transferases:the first enzymatic in mercapturic and formation. Journal of biology chemistry. 249,7130-7139.

Halliwell, B. (1994). Free radicals, antioxidants and human disease: curiosity, cause or consequence. Lancet 344, 721–724

Halliwell, B., Gutteridge, C., (1999). Free Radicals in Biology and Medicine. New York Oxford University. 36

Harris, G. (1973). Nudibranch associations. In: Current Topics in Comparative Pathobiology. 2, 213-315. Academic Press.

Hebbel, P. (1986), Erythrocyte antioxidants and membrane vulnerability. Journal of Laboratory and Clinical Medicine 107(5), 401-404

Henson, L., Stauffer, G., Gallagher, P. (2001). Induction of glutathione S-transferase activity and protein expression in brown bullhead (*Ameiurus nebulosus*) liver by ethoxyquin. Toxicological sciences. 62(1), 54-60.

Hermes-Lima, M., Moreira, C., Rivera-Ingraham, G., Giraud-Billoud, M., Genaro-Mattos, C., Campos, G. (2015). Preparation for oxidative stress under hypoxia and metabolic depression: Revisiting the proposal two decades later. Free Radical Biology and medicine. 89, 1122-1143

Hermes-Lima, M., Storey, J., Storey, K. (1998). Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. Comparative Biochemistry and Physiology. Part B. 120, 437-448

Hermes-Lima, M., Zenteno-Savín, T. (2002). Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comparative Biochemistry and Physiology. Part C. 133, 537-556

Hirose, M., Hirose, E., Kiyomoto, M. (2015). Identification of five species of Dendrodoris (Mollusca: Nudigills) from Japan, using DNA barcode and larval characters. Marine Biodiversity. 45(4), 769-780.

Hochachka, E., Somero, N. (2002). Biochemical adaptation: Mechanisms and Process in Physiological Evolution. Oxford University Press, Oxford

Hochachka, W., Buck, T., Doll, J., Land, C., (1996) Unifying theory of hypoxia tolerance: Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proceedings of the National Academy of Sciences of the United States of America. 93, 9493-9498

Hoover, R., Armour, R., Dow, I., Purcell, J. (2012). Nudibranch predation and dietary preference for the polyps of *Aurelia labiata* (Cnidaria: Scyphozoa). Hydrobiologia. 690,199–213

Huggett, J., Kimerle, A., Mehrle-Jr., M., Bergman, L., (1992). Biomarkers: biochemichal, physiological, and histological markers of anthropogenic stress. Lewis Publisher, Chelsea.

Jensen, C., Armstrong, A., (1991) Intertidal zonation among congeners: factors regulating distribution of porcelain crabs *Petrolisther spp.* (Anomura: Porcellanidae) Marine ecology progress series. 73, 47-60.

Lesser, P., (2006).Oxidative stress in marine environments: biochemistry and physiological ecology. Annual Review Physiology. 68, 253-278

Lesser, P., (2011). Oxidative stress in tropical marine ecosystems. Oxidative Stress in Aquatic Ecosystems. John Wiley & Sons Ltd, 7-19.

Madeira, D., Narciso, L., Cabral, H., Vinagre, C., Diniz, M. (2013). Influence of temperature in thermal and oxidative stress responses in estuarine fish. Comparative Biochemistry and Physiology. Part A. 166(2), 237-243.

Matoo, O., Ivanina, A., Ullstad, C., Beniash, E., Sokolova, I. (2013). Interactive effects of elevated temperature ans CO_2 levels on metabolism and oxidative stress in two common marine bivalves (*Crassostrea virginica* and *Mercenaria mercenaria*). Comparative Biochemistry and Physiology. Part A 164, 545-553.

Milton, S., Nayak, G., Kesaraju, S., Kara, L., Prentice, H. (2007). Suppression of reactive oxygen species production enhances neuronal survival in vitro and in vivo in the anoxia-tolerant turtle *Trachemys scripta*. Journal of Neurochemistry. 101(4), 993-1001.

Njemini, R., Demanet, C., Mets, T. (2005) Comparison of two ELISAs for the determination of Hsp70 in serum. Journal Immunology Methods. 306, 176-182.

Nybakken, J. (1978). Abundance, diversity and temporal variability in a California intertidal nudibranch assemblage. Marine Biology 45(2): 129-146.

Nybakken, J. (1988). Marine Biology: An Ecological Approach. Harper-Collins, New York

Paine, T., (1974). Experimental studies on the relationship between a dominant competitor and its principal predator. 15, 93-120.

Pelham, B. (1986). Speculations on the functions of the major heat shock and glucose-regulated proteins. Cell. 46(7), 959-961.

Potts, W., (1970). The ecology of *Onchidoris fusca* (Nudigills). Journal of the Marine Biological Association of the United Kingdom. 50, 269-292.

Potts, W., (1983). The respiration of *Onchidoris bilamellata* and *Arquidoris pseudoargus* (Doridacea). Journal of the Marine Biological Association of the United Kingdom. 63, 399-407.

Regoli, F., Pellegrini, D., Winston, W., Gorbi, S., Giuliani, S., Virno-Lamberti, C., Bompadre, S. (2002). Application of biomarkers for assessing the biological impact of dredged materials in the Mediterranean: the relationship between antioxidant responses and susceptibility to oxidative stress in the red mullet (Mullus barbatus). Marine Pollution Bulletin. 44, 912–922.

Rivera-Ingraham, A., Bickmeyer, U., Abele, D. (2013). The physiological response of the marine platyhelminth *Macrostomum lignamo* to different environmental oxygen concentrations. Journal of Experimental Biology. 216, 2741-2751.

Rivera-Ingraham, A., Rocchetta, I., Meyer, S., Abele, D. (2013). Oxygen radical formation in anoxic transgression and anoxia-reoxygenation: foe or phantom? Experiments with a hypoxia tolerant bivalve. Marine Environmental Research. 92. 110-119

Rosa, R., Seibel, A. (2008) Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. Proceedings of the National Academy of Sciences of the United States of America. 105, 20776-20780.

Rosa, R., Seibel, A. (2010). Metabolic physiology of the Humboldt squid. *Dosidicius gigas*: implications for vertical migration in a pronounced oxygen minimum zone. Progress Oceanography. 86, 72-80

Sagasti, A., Schaffner, L., Duffy, J. Effects of periodic hypoxia on mortality, feeding and predation in an estuarine epifaunal community. Journal of Experimental Marine Biology and Ecology. 257–283

Sies, H. (1993). Strategies of antioxidant defenses. European Journal Biochemistry, 215, 213-219.

Sisson, G. (2005). Life history dynamics and biogeography of a nudibranch with contrasting developmental modes: A hypothesis for the evolution of larval types. Journal of Natural History. 39(20), 1719-1733.

Sokolova, M., Sukhotin, A., Lannig, G., (2011). Stress effects on metabolism and energy budgets in mollusks. Oxidative Stress in Aquatic Ecosystems. 263-280.

Stillman, J., (2002). Causes and consequences of thermal tolerance limits in rocky interdital porcelain crabs, Genus *Petrolishes*. Integrative of Comparative Biology. 42, 790-796.

Storey, B., Storey, M. (2004). Metabolic rate depression in animals: transcriptional and translational controls. Biological Reviews 79(1), 207-233.

Sun, Y., Oberley, L., Li, Y., (1988). A simple method for clinical assay of superoxide dismutase. Clinical Chemistry 34(3), 497–500

Teixeira, T., Diniz, M., Calado, R., Rosa, R. (2013). Coral physiological adaptations to air exposure: Heat shock and oxidative stress responses in *Veretillum cynomorium.* Journal of Experimental Marine Biology and Ecology. 439, 35-41.

Todd, D. (1981). The ecology of nudibranch molluscs. Oceanography and Marine Biology Annual Review. 19, 141-234.

Todd, D. (1983). Reproductive and trophic ecology of nudibranch molluscs. In: Russel-Hunter, D., (Ed.) The molluscs. 6, 225-259. Academic Press, London.

Tomanek, L., (2010). Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographic distribution ranges and metabolic costs. Journal of Experimental Biology. 213, 971-979.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic

environmental pollutants. Ecotoxicology and Environmental Safety. 64, 178–89.

Valdés, A., (2000). *Dendrodoris herytra* Valdés & Ortea, 1996 . *[In] Sea Slug Forum.* Australian Museum, Sydney.

Valdés,Á., Ortea, J., Ávila, C., Ballesteros, M. (1996). Review of the genus *Dendrodoris Ehrenberg*, 1831 (Gastropoda:Nudigills) in the Atlantic Ocean. Journal of Molluscan Studies.62.1-31

Welker, A., Moreira, D., Campos, É., Hermes-Lima, M. (2013). Role of redox metabolism for adaptation of aquatic animals to drastic changes in oxygen availability. Comparative Biochemistry Physiology. Part A. 165, 384-404

Willmer, P., Stone, G., Johnston, I. (2005). Environmental Physiology of Animals. Blackwell Science. Oxford

Yamashita, M., Yabu, T., Ojima, N. (2010). Stress Protein HSP70 in Fish. Aqua-BioScience Monographs. 3(4), 111-141

Yonow, N. (2015). Sea Slugs: Unexpected Biodiversity and Distribution. In: Rasul, N., Stewart, I. (Ed) The Red Sea. 531-550.

Zenteno-Savin, T. (2002). Animal response to drastic changes in oxygen availability and physiological oxidative stress. Comparative Biochemistry and Physiology. Biochemistry and Molecular Biology. Part B. 537–556.